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13. Abstract *(Maximum 200 Words)* ***(abstract should contain no proprietary or confidential information)***

Mammographic density appears to be a strong predictor of breast cancer risk. Endogenous steroid hormones have been shown to be important in the development and progression of breast cancer. Because some metabolites of endogenous estrogens may have more estrogenic effects than others, genetically determined differences in biosynthesis and metabolic pathways of estrogens may affect breast cancer risk. This study will address the following specific aims: 1. To examine the relation between the urinary ratio of 16α-hydroxyestrone (16α-OHE1) and 2-hydroxyestrone (OHE1) and polymorphisms in the genes coding for some hormone metabolizing enzymes. 2.To investigate differences in urinary excretion of estrone-3-glucuronide, a marker of endogenous estradiol, between women with variant alleles for polymorphisms in genes coding for hormone producing (*CYP17*) and metabolizing enzymes (*COMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1*) and women with the wildtype genotype. 3. To analyze the relation of mammographic density patterns with the urinary excretion of estrone-3- glucuronide, the urinary ratio of 16α-OHE1: 2-OHE1, and the presence of variant alleles for hormone producing and metabolizing enzymes.

During the last year, we have completed the recruitment of 316 study subjects (218 pre- and 98 postmenopausal women). The subjects represent the population of Hawaii with large proportions of women from different ethnic groups. The majority of mammograms has undergone quantitative mammographic density assessment. Blood for the DNA analysis and urine samples for the premenopausal women were collected on cycle day 19. Blood or mouthwash and urine samples were collected for all women, coded, processed, and frozen. The genotyping for 7 polymorphisms using published PCR/RFLP methods has just been completed, 2 more are under way. The urinary analysis will be finished within the next 2 months. The preliminary analysis of the relation between ethnicity and variant alleles suggests several differences in genotype by ethnic background. Associations between mammographic density and the presence of variant alleles appear to exist for several enzyme coding genes and will be explored further in regression models with the adjustment for confounders.

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(4) Introduction

The focus of this study is to identify genetic characteristics that may put women at higher risk to develop breast cancer. Recent research has identified variant alleles in genes that code for common enzymes involved in the biosynthesis and metabolism of endogenous estrogens. In contrast to the low population prevalence of mutations in *BRCA1* and *BRCA2*, variant alleles in these enzyme coding genes are very common (up to 50% prevalence in some populations). Because of their high prevalence, they have the potential to play an important role in breast cancer etiology even if the risk related to these genes is relatively low. It is possible that a large number of women carry variant alleles that predispose them to develop breast cancer.

The two major innovative features of our project are the simultaneous investigation of several enzyme coding genes and the fact that we examine the relation of genes coding for hormone metabolizing enzymes to probable predictors of breast cancer, urinary estrone-3-glucuronide excretion, the ratio of different estrogen metabolites, and mammographic densities. In combination with our recruitment strategy, i.e., asking women from population-based studies to donate a DNA sample, this project has the ability to assess several polymorphisms at once and to examine their relation to predictors of breast cancer at a relatively low cost. With few exceptions, the polymorphisms to be investigated in this project have not been examined among women of Asian and Pacific Islander ancestry whose breast cancer risk is lower than among Caucasian women. More than 50% of our study participants will be non-Caucasians. Therefore, the proposed study has the potential to generate new information on the prevalence of variant alleles among these ethnic groups and their possible associations with breast cancer risk.

Mammographic density appears to be a strong predictor of breast cancer risk, conferring a fourfold risk when women with high-density patterns are compared to women with low-density patterns. Endogenous steroid hormones have been shown to be important in the development and progression of breast cancer. Because some metabolites of endogenous estrogens may have more estrogenic effects than others, genetically determined differences in biosynthesis and metabolic pathways of estrogens may affect breast cancer risk. The metabolism of estradiol follows 2 major competing pathways, C2- and C16α-hydroxylation, and a minor C4-hydroxylation. Whereas the 2-hydroxylated metabolites have only weak peripheral estrogenic activity, the 16α-hydroxylated and probably the 4-hydroxylated metabolites are strong estrogen agonists.

This study addresses the following specific aims:

1. To examine the relation between the urinary ratio of 16α-hydroxyestrone (16α-OHE1) and 2-hydroxyestrone (2-OHE1) and polymorphisms in the genes coding for hormone metabolizing enzymes *COMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1*.
2. To investigate differences in urinary excretion of estrone-3-glucuronide, a marker of endogenous estradiol, between women with variant alleles for polymorphisms in genes coding for hormone producing (*CYP17*) and metabolizing enzymes (*COMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1*) and women with the wildtype genotype.
3. To analyze the relation of mammographic density patterns with the urinary excretion of estrone-3-glucuronide, the urinary ratio of 16α-OHE1 : 2-OHE1, and the presence of variant alleles for hormone producing and metabolizing enzymes.

In addition to the originally proposed polymorphisms, we decided to analyze genotypes for two other enzymes that have been described in the literature as possibly related to breast cancer risk: Growth Hormone-1 (*GH-1*) and UDP Glucuronosyl Transferase 1A *(UGT1A1)*(1).

(5) Body

A. Recruitment and Data collection

During this year, we have accomplished the following tasks outlined in the approved Statement of Work.

Task 1. This work had been completed during the first year.

Task 2. Collection of specimens for 150 postmenopausal women from previous study (Months 4-15).
Recruitment. The recruitment of pre-and postmenopausal women (N = 316) was completed by the end of year 2001. Eligible postmenopausal women were identified from the previous study "Ethnicity, Soybean Consumption, and Mammographic Density". Invitation letters explaining the new study were mailed to 345 prospective study participants. Four waves of mailing were completed at one-month intervals. To increase prospective participants' interest in the study, abstracts of articles published as a result of our previous studies were mailed along with the invitation letter. The prospective participants were asked to call the project coordinator and set up an appointment to come to the Cancer Research Center. In case this was impossible, a meeting at another location was arranged. During the meeting, the project coordinator explained the study goals and procedures, answered questions regarding the study, and obtained written informed consent in a presence of an independent witness. One copy of the consent form was given to the participant for her own records; the other copy is kept at the Cancer Research Center. As a result, we have recruited 98 eligible postmenopausal women who had participated in the earlier study. The mean age of postmenopausal study participants was 60.08±8.26 years. The ethnic distribution of the entire study population (N=298) for whom we have complete data at this time is shown in Table 1. More than half of the participants (56.7%) have ancestries other than Caucasian.

Collection of specimens. Urine samples were collected for all 98 recruited subjects at the time of the appointment, using sterile disposable equipment. For each woman, 20 ml of urine were aliquoted into ten 2ml cryogenic vials that were labeled and stored in the ultra-low freezer at - 80^{0}C. Blood samples were collected for 65 participants. The blood collection procedure was established according to the changes in the protocol approved by the U.S. Army Research and Material Command (May 31, 2001) and the University of Hawaii Committee on Human Studies (June 29, 2001). All blood collecting and processing was performed by a certified phlebotomist, using sterile disposable equipment, at the Queens Medical Center laboratory. Blood was transported by trained personnel, according to the applied regulations approved by the University of Hawaii Institutional Biosafety Committee on 04/02/01. A sample of 4 ml blood was drawn into vacutainer with EDTA, aliquoted into 1.2 ml cryogenic vials and frozen at - 80^{0}C. In addition,10 ml of blood were drawn into vacutainer without any additives, centrifuged at 2200 rpm for 15 minutes; serum was aliquoted into five 1.2 ml cryogenic vials and stored in the ultra-low freezer at - 80^{0}C. For participants who declined a blood draw, we collected buccal cells by asking them to swish mouthwash in their mouth for 60 seconds and to expel it in a collection container. The procedure allows isolation of a sufficient amount of DNA for genotyping. Overall, 33 participants preferred the mouthwash procedure to a blood draw; buccal cells were collected from them and stored at - 80^{0}C.

Task 3. Recruit 150 premenopausal women who participate in the "Effects of Soy on Estrogens and Mammographic Densities" study.
Recruitment. Premenopausal women who are participating in the ongoing intervention "Effects of Soy on Estrogens and Mammographic Densities" were recruited at the time of their visit to the Cancer Research Center. Written informed consent was obtained from all women who decided to participate in this study. During this year, we have recruited 68 premenopausal women in addition to the 150 recruited during the first year. The premenopausal group consists of 218 women with a mean age 43.9± 2.8 years. The subjects represent the population of Hawaii with large proportions of women of Japanese, Filipino, and Native Hawaiian ancestry (Table 1). Given the successful recruitment of more than 200 premenopausal women, we reached our recruitment goal of 300 although the number of postmenopausal women did not reach 150.

Table 1. Characteristics of 298 Women in the Genetic Polymorphism Study

Variable		Mean	Std Dev
Age (years)		46.2	8.0
Breast area (pixels)		238,163	126,191
Dense area (pixels)		64,600	40,245
Percent density		31.2	17.1
Body mass index		26.0	5.7
Body weight		66.9	16.2
Height		1.6	0.1
Daily energy intake (kcal)		1925	830
Percent calories from fat		31.5	6.1
		Number	**Percent**
Ethnicity	Caucasian	129	43.3
	Chinese	30	10.1
	Filipino	12	4.0
	Hawaiian	37	12.4
	Japanese	67	22.5
	Other	23	7.7

Collection of specimens. Plasma estrogen levels vary considerably during the menstrual cycle in premenopausal women. Therefore, it was crucial to collect samples at a defined time during the menstrual cycle. The collection was timed with the help of an ovulation kit. Based on information about previous cycle length, the women started using an ovulation predictor several days before their expected ovulation. This kit detects the mid-cycle rise of luteinizing hormone (LH) using morning urine. It is sensitive to 35 mIU/mL of LH, simple to use, and considerate 99% accurate in predicting ovulation. Samples for all premenopausal women, collected on approximately day 19 of a 28-day cycle are available for this study. The urine specimens were processed and stored in the ultra-low (- 80^{0}C) freezer. Blood and serum samples will be also available for all recruited premenopausal women. All collected urine, whole blood, and serum samples are stored in the cryogenic vials labeled with subject's numeric code and the date of collection in the designated racks in the ultra-low freezer (- 80^{0}C). Storage information was entered into the computerized study database and duplicated in the specimens' log journal. Each subject in the study has been assigned a numeric code to allow linkage of different data elements. The study database will not contain names or other identifying information. Mammograms have been obtained for 95% of the premenopausal women, the remaining films are currently being retrieved from mammography clinics.

Task 4. Performing genotyping.

We proceeded with this task in October of 2001. The frozen blood and buccal cell samples were removed from storage for DNA extraction. DNA was extracted by a standard method and analyzed by published PCR/RFLP methods for the presence of the variant alleles for the genes under study. By the end of July 2002, the following genotyping analyses had been completed for the majority of study participants: *GH-1*, *COMT*, *Cyp17-MspA1*, *Cyp1B1-Pst*, *Cyp1A1 - exon7 - BsrDI* , *Cyp1A1 - exon 7 – Bsa*, *Cyp1A1-Msp*. The laboratory is still working on two more polymorphisms: *UGT1A* and *Cyp1A2 – intron1*. These genotyping will be completed by the end of 2002.

Task 5. Performing urinary analysis for estrogen metabolites
All frozen urine samples were shipped on dried ice to the hormone laboratory. Urinary levels of estrone-3-glucuronide, 16α-OHE1, and 2-OHE1 will be assessed using an enzyme immunoassay. The analysis will be completed by the end of September 2002.

Task 6. Data management and analysis
The majority of mammograms have been scanned and quantitative density assessment has been performed. Results of genotyping, urinary analyses, and mammographic density assessment information have been entered into the study database. The preliminary examination of the data has been initiated. The analysis is being performed using the SAS statistical software package (SAS Institute Inc., Cary NC) for PC. We examined the distribution of the variant alleles by ethnicity using chi-square tests (Table 2). To test the relation of variant alleles with mammographic densities, we applied analysis of variance (ANOVA). In the future, we will apply multiple linear regression models, using the stepwise selection method to examine the combined effect of several variant alleles on mammographic densities and urinary estrogen metabolites. This approach will allow us to build statistical models that fit more than one polymorphism and to adjust for known confounders.

B. Preliminary Results

Since the genotyping was just finished, only very few statistical analyses have been performed. In relation to ethnicity, we noticed significant differences for several polymorphisms (Table 2). The mutant allele in the gene coding for *COMT* appears more common among Caucasians than among all other groups. The same appears to be true for the mutant allele of *Cyp1B1-Pst*. On the other hand, for *Cyp1A1 - exon7 – BsrDI* and for *Cyp1A1-Msp*, the wildtype was observed more frequently among Caucasian women. The other distributions did not show significant differences by ethnic group.

Looking at mean mammographic density by genotype without adjustment for other determinants of mammographic density, such as body mass index or reproductive factors (Table 2), two significant relations emerge: *Cyp1A1 - exon7 – BsrDI* and *Cyp1B1-Pst*. For *Cyp1A1 - exon7 – BsrDI*, mammographic density is 29.4% for the wildtype, 33.6% for the heterozygote, and 39.7% for the mutant genotype. In agreement with our findings, one previous study described an association between breast cancer risk and the presence of the mutant alleles among African-American women (2). However, another case-control study (3) did not find a relation. For *Cyp1B1-Pst*, the relation is in the opposite direction as for *Cyp1A1*. Percent density for the wildtype is the highest (34.9%), intermediate (29.3%) for the heterozygote, and lowest (24.6%) for the mutant. No consistent association was found in case-control studies that investigated the relation with breast cancer (4;5). Another interesting relation is apparent for *COMT* although it is not statistically significant. Mammographic density is highest for the wildtype (32.2%), intermediate for the heterozygote (31.4%), and lowest for the mutant (28.1%). However, the relation is opposite to the hypothesis that women with the mutant genotype with a low activity enzyme experience a lower breast cancer risk than women with the wildtype allele (6;7). The results for *Cyp17-MspA1* genotype are also not statistically significant, but the weak trend 29.3% for the wildtype, 31.4% for the heterozygotes, and 34.3% for the mutant genotype is in agreement with some previous studies that described a higher breast cancer risk for women with the mutant alleles (8;9). For *GH-1* and for the *Cyp1A1 – exon 7 – Bsa* marker, mammographic density does not appear to differ by genotype. In more detailed analyses, we will explore these relations after adjustment for age, ethnicity, menopausal status, body mass index, and reproductive characteristics. These variables are known to be strongly related to mammographic density and may confound the relation with genotype.

Table 2. The Relation of Mammographic Density and Ethnicity with Genotypes

Polymorphism	Type	Percent density	N	p for ANOVA	Ethnicity						p for chi-Square
					C	F	H	J	O	W	
GH-1	**AA**	30.1	51	0.59	8 26.7	3 25.0	6 16.7	5 7.5	3 13.0	26 20.5	0.14
AA=Mutant											
AT=Heterozygote	**AT**	32.1	143		15 50.0	6 50.0	14 38.9	39 58.2	16 69.6	59 46.5	
TT=Wildtype											
	TT	30.1	91		7 23.3	3 25.0	16 44.4	23 34.3	4 17.4	42 33.0	
COMT	**HH**	32.2	109	0.34	9 30.0	8 66.6	25 67.6	38 56.7	6 26.1	28 21.9	<0.0001
HH=Wildtype											
HL= Heterozygote	**HL**	31.4	124		19 63.3	4 33.3	9 24.3	25 37.3	14 60.9	57 44.5	
LL=Mutant											
	LL	28.1	54		2 6.7	0 0.0	3 8.1	4 5.9	3 13.0	43 33.6	
Cyp17-MspA1	**M**	34.3	44	0.25	6 20.0	1 8.3	5 13.5	13 19.4	3 13.0	17 13.3	0.93
M=Mutant											
H=Heterozygote	**H**	31.4	140		15 50.0	6 50.0	17 46.0	35 52.2	12 52.2	61 47.7	
W=Wildtype											
	W	29.3	103		9 30.0	5 41.7	15 40.5	19 28.4	8 34.8	50 39.1	
Cyp1B1-Pst	**LL**	34.9	121	0.0026	16 53.3	6 50.0	15 40.5	41 61.2	9 40.9	39 30.7	0.006
LL=Wildtype											
LV=Heterozygote	**LV**	29.3	135		12 40.0	6 50.0	21 56.8	22 32.8	11 50.0	68 53.5	
VV=Mutant											
	VV	24.6	29		2 6.7	0 0.0	1 2.7	4 6.0	2 9.1	20 15.8	
Cyp1A1 - exon7 - BsrDI	**AA**	29.4	185	0.02	21 72.4	7 63.6	23 62.2	30 46.2	14 60.9	97 77.6	0.005
AA=Wildtype											
AG=Heterozygote	**AG**	33.6	79		8 27.6	3 27.3	13 35.1	28 43.1	6 26.1	24 19.2	
GG=Mutant											
	GG	39.7	16		0 0.0	1 9.1	1 2.7	7 10.8	3 13.0	4 3.2	
Cyp1A1 - exon 7 – Bsa	**AA**	38.0	1	0.40	0 0.0	0 0.0	1 2.7	0 0.0	0 0.0	0 0.0	0.28
AA=Mutant											
CA=Heterozygote	**CA**	25.5	15		2 6.7	0 0.0	1 2.7	2 3.0	0 0.0	10 7.8	
CC=Wildtype											
	CC	31.5	270		28 93.3	12 100.0	35 94.6	64 97.0	23 100.0	118 92.2	
Cyp1A1-Msp	**A**	29.9	140	0.18	13 44.8	3 27.3	14 38.9	21 32.3	13 56.5	80 65.6	0.0002
A=Wildtype											
B=Heterozygote	**B**	31.8	102		14 48.3	6 54.6	15 41.7	29 44.6	5 21.7	36 29.5	
C=Mutant											
	C	35.9	34		2 6.9	2 18.2	7 19.4	15 23.1	5 21.7	6 4.9	

No relation between CYP17 polymorphism and mammographic density

Abstract for AACR 2002 Conference on Frontiers in Cancer Prevention Research

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The cytochrome P450c17alpha enzyme (CYP17) plays a role in the formation of precursor androgen steroids that can be transformed to active estrogens. The presence of a common variant allele of the CYP17 gene, designated A2, has been associated with breast cancer risk as well as with higher serum estradiol and progesterone levels in several studies. It has been proposed that the variant A2 allele may result in an increased rate of transcription leading to increased estradiol production. Mammographic density is a strong predictor of breast cancer risk and may be determined by circulating hormones. Based on the hypothesis that the variant allele may be associated with higher breast cancer risk, the objective of this study was to analyze the relation of the CYP17 A2 allele with mammographic density. We recruited premenopausal healthy women from mammography clinics. All subjects had a normal mammogram, completed a dietary and a medical history, and donated a blood sample. DNA was extracted from whole blood with a rapid method using proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The DNA samples were analyzed by a PCR/RFLP method for the presence of the variant allele in CYP17 (MspAI). After digitizing the mammographic films, the images were assessed for densities with a computer-assisted method. Percent density was calculated as the ratio of the dense areas over the total area of the breast. We applied analysis of variance to test for associations between genotype and percent mammographic density. The study population for this analysis included 192 women (75 Caucasian, 47 Japanese, 24 Hawaiian, 18 Chinese, 12 Filipino, and 16 other ethnicity). The mean age was 43.1 years (range: 35-47 years) and the mean body mass index was 26.5 kg/m2 (range: 17.7-49.2). In this population, 71 (37%) women carried two wildtype alleles, 95 women (49.5%) were heterozygous, and 26 (13.5%) women carried two variant alleles. Genotype was not significantly associated with ethnic background and body weight. However, height differed significantly by genotype ($p = 0.04$) with a mean of 1.61 m for A1/A1, 1.59 m for A1/A2, and 1.57 m for A2/A2. Mean percent density was 30.7%, 30.5%, and 32.1% ($p = 0.91$) for the homozygous wildtype, heterozygous, and homozygous variant genotype, respectively. Inclusion of potential covariates (body mass index, age, and ethnicity) did not change this relation. In conclusion, the presence of the putative high risk CYP17 A2 allele was not related to mammographic density in a population of premenopausal women.